

Different Genotypes of Human Polyomaviruses Found in Patients With Autoimmune Diseases in Taiwan

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We have assayed for the presence of human polyomaviruses in urine of autoimmune disease patients, such as systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), rheumatoid arthritis (RA), or dermatomyositis/polymyositis (DM/PM), by PCR. The results indicate that approximately 40% of patients were JCV positive and 15% of the JCV positive patients were also infected by BKV at the same time according to Southern blot and DNA sequencing of the PCR products. Interestingly, the JCV present in autoimmune diseases patients were Taiwan-1, Taiwan-2, and Taiwan-3 strains with pentanucleotide-A (GGGAA) and/or -B (AAAGC) deletions within the regulatory region. In addition, BKV found in the examined samples were Taichung-1 and Taichung-2 strains. Taichung-1 had two nucleotide alterations and Taichung-2 had six nucleotide differences within the regulatory region when compared to WW BKV archetype. Although the examined autoimmune diseases patients included RA, SLE, PM, DM, and SS patients, there appears to be no correlation between disease and virus strains. However, Taiwan-2 strain JCV with two copies of pentanucleotide-A deletion was present in the patient with the longest period of immunosuppressive medication.

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KEY WORDS: JC virus, BK virus, immunosuppression

INTRODUCTION

Human polyomaviruses JC virus (JCV) and BK virus (BKV) are ubiquitous in the human population [Walker and Frisque, 1986]. The viruses may asymptotically persist in renal tissue [Chesters et al., 1983; Walker and Frisque, 1986] and may excrete in urine [Kitamura et al., 1990, 1994; Markowitz et al., 1991]. However, JCV can cause a fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML) [Padgett and Walker, 1973; Padgett et al., 1976, 1977], and BKV may cause hemorrhagic cystitis [Apperley et al., 1987; Arthur et al., 1986] by unknown mechanism(s).

It has been speculated that JCV may persist in the kidney in a form termed archetype [Yogo et al., 1990]. During the long term immunosuppressive condition, the virus may be reactivated and may rearrange its promoter-enhancer regulatory region by deletion and/or duplications to form different strains and cause PML in brain [Chuke et al., 1986; Dorries, 1984; Frisque et al., 1984; Martin and Foster, 1984; White et al., 1992]. This speculation could be correlated with the fact that about 5% of AIDS patients succumb to PML [Berger et al., 1987; Krupp et al., 1985; Kure et al., 1991; Snider et al., 1983].

Patients with various autoimmune diseases, such as systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), rheumatoid arthritis (RA), or dermatomyositis/polymyositis (DM/PM), primarily become immunocompromised because of intrinsic abnormalities of the immune system, or immunosuppressive therapy. Immunocompromised conditions can reactivate human polyomaviruses JCV and BKV, and lead to viruria [Arthur et al., 1988; Coleman et al., 1980; Gardner et al., 1984; Myers et al., 1989; Yogo et al., 1991].

In this study we tried to determine the presence of human polyomavirus in urine of autoimmune disease patients by PCR. The genotypes of JCV present in the examined patients were Taiwan-1 (TW-1), Taiwan-2 (TW-2), and Taiwan-3 (TW-3) with pentanucleotide-A (GGGAA) and/or pentanucleotide-B (AAAGC) deletions within the regulatory region. Taichung-1 (TC-1) with two nucleotide alterations and Taichung-2 (TC-2) with six nucleotide alterations when compared to WW BKV archetype were found in the examined samples. It was found that approximately 40% of the examined urine samples were JCV positive and 15% of the samples were both JCV and BKV positive.

MATERIALS AND METHODS

Sample Collection

Patients with various autoimmune diseases were consecutively enrolled in this study. Patients with systemic

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lupus erythematosus were diagnosed according to the 1982 revised criteria of American Rheumatism Association (ARA) for the classification of SLE [Tan et al., 1982]; patients with rheumatoid arthritis, according to the 1987 revised ARA criteria for classification of RA [Arnett, 1988]; and patients with DM/PM according to the diagnostic criteria of Bohan and Peter [1975]. SS was established by a history of dry eye and mouth, positive Shirmer's test, corneal punctate erosions, and lip biopsy. Urine specimens were collected at the Hospital of Chung Shan Medical and Dental College. All of samples were immediately frozen at -20°C after collection until analyzed.

Sample Preparation

Ten milliliters of urine were ultracentrifuged at $142,000g$ for 90 min at 4°C using a Beckman SW 41Ti rotor in a Beckman L8-70M ultracentrifuge. The sedimented pellet was resuspended in 1 ml of distilled water by brief vortexing. Five microliters of the resuspended fluid was mixed with 4 μl of water and 1 μl of $10 \times$ lysis buffer containing proteinase K (100 mM Tris-HCl, 10 mM EDTA, pH 8.0 and 500 $\mu\text{g/ml}$ proteinase K) to lyse cells. The mixture was incubated at 50°C for 15 min and then at 95°C for 10 min, and centrifuged at 10,000 rpm for 3 min. The supernatant was collected and used for polymerase chain reaction.

Polymerase Chain Reaction

Nine microliters of the supernatant from the lysed mixture was used for viral DNA amplification in a total volume of 50 μl by polymerase chain reaction (PCR). The PCR reagents contained 20 pmole of each primer, JBR1 (5'-CCTCCACGCCCTTACTACTTCTGAG-3') and JBR2 (5'-GTGACAGCTGGCGAAGAACCATGGC-3') [White et al., 1992], 5 μl of $10 \times$ Taq DNA polymerase buffer 1.5 mM MgCl_2 , 0.2 mM of each dNTP, and 2 units of Taq DNA polymerase (Promega, WI). The PCR reaction mix was overlaid with 50 μl of mineral oil (Sigma, MO). After 30 sec centrifugation at 10,000 rpm, the PCR reaction was followed. The initial step of PCR was 95°C for 3 min and then 40 cycles of 95°C for 45 sec (denaturation), 55°C for 2 min (annealing), and 72°C extension for 2 min were followed by 72°C for 4 min to extend the last cycle using a Perkin-Elmer Cetus DNA Thermal cycler.

PCR Products Analysis by Electrophoresis

Five microliters of the PCR reaction mix of each sample was loaded into a 1.5% agarose (molecular biology grade; IBI Biotechnologies, CN) gel and subjected to electrophoresis in TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM sodium borate, 2.5 mM EDTA) at 80 volts for 30 min. The agarose gel was stained with ethidium bromide (0.5 $\mu\text{g/ml}$) for 5 min to visualize the band and then photographed under UV light.

Southern Blot of PCR Product

Ten microliters of PCR reaction mixture was electrophoresed in a 1.5% agarose gel in TAE buffer (40 mM

Tris-acetate, pH 8.5, 2 mM EDTA). For Southern blot, the DNA fragments in the agarose gel were transferred onto nylon membrane (MSI, MA) by using semi-dry Horizblot (ATTO Corp., Tokyo, Japan) at 3 mA per cm^2 of nylon membrane for 3 hr. The detail protocols were provided by the company. The membrane was initially treated with an alkaline solution (0.2 N NaOH and 0.6 M NaCl) for 1 hr and then treated with Tris buffer (1.0 M Tris, pH 7.4 and 0.6 M NaCl) for 1 hr. Prehybridization was followed by treating the membrane with $5 \times$ Denhardt's solution (20 μg each of bovine serum albumin [Sigma], Ficoll [Pharmacia, NJ], and polyvinylpyrrolidone [Sigma] per ml) and $6 \times$ SSC (0.9 M NaCl and 0.09 M sodium citrate) at 58°C for 5 hr. The solution was replaced with a fresh hybridization solution of $5 \times$ Denhardt's, $6 \times$ SSC, and 0.5% sodium dodecyl sulfate (SDS) containing 4×10^5 cpm/ml of (^{32}P) ATP-end-labeled JBR-3 oligonucleotide probe (5'-TGGCAGTTA-TAGTGAAACCCC-3'). Following 5 hr hybridization at 58°C with shaking, the Nylon membrane was washed five times with $2 \times$ SSC solution with 1.0% SDS at 58°C for 3 min each and five times with $1 \times$ SSC solution with 1.0% SDS at 58°C for 3 min each and five times with $0.2 \times$ SSC solution with 1.0% SDS at 58°C for 3 min each wash. The membrane was air dried and exposed on Kodak X-AR film for overnight at -70°C .

DNA Sequencing

Thirty microliters of PCR reaction mixture of the JCV or BKV positive samples was loaded on a 1.5% low melting agarose (Promega) gel in TAE buffer for electrophoresis. The band containing the DNA fragment was visualized by staining the gel with ethidium bromide and excised under UV light. The DNA fragment in the low melting agarose was purified by Magic PCR Minipreps kit (Promega). The purified DNA fragment was directly sequenced by the fmol DNA sequencing kit purchased from Promega. For reading the JCV and BKV mixed DNA sequences, JCV CY archetype sequences [Yogo et al., 1990] were used to mark JCV sequences on the X-ray film and then BKV WW archetype sequences [Rubinstein et al., 1987] were used to read the remaining BKV sequences on the film.

RESULTS

PCR Amplification of Viral DNA

Twenty urine samples were collected from patients with various autoimmune diseases including 11 rheumatoid arthritis (RA), two Sjogren's syndrome (SS), three systemic lupus erythematosus (SLE), two polymyositis (PM), and one dermatomyositis (DM). The primers, JBR1 and JBR2, were used to amplify the viral DNA regulatory region in PCR. The primers were flanking the constant ends of regulatory region to generate a 334 base pairs fragment from nucleotide -45 to $+289$. After 40 cycles of PCR, the reaction mixture was analyzed by agarose electrophoresis. The results showed that eight of 20 samples contained viral DNA (Fig. 1, lanes 4, 5, 7, 12, 14, 15, 16, and 18). The size of the DNA fragments generated by JBR1 and JBR2 primers were expected to

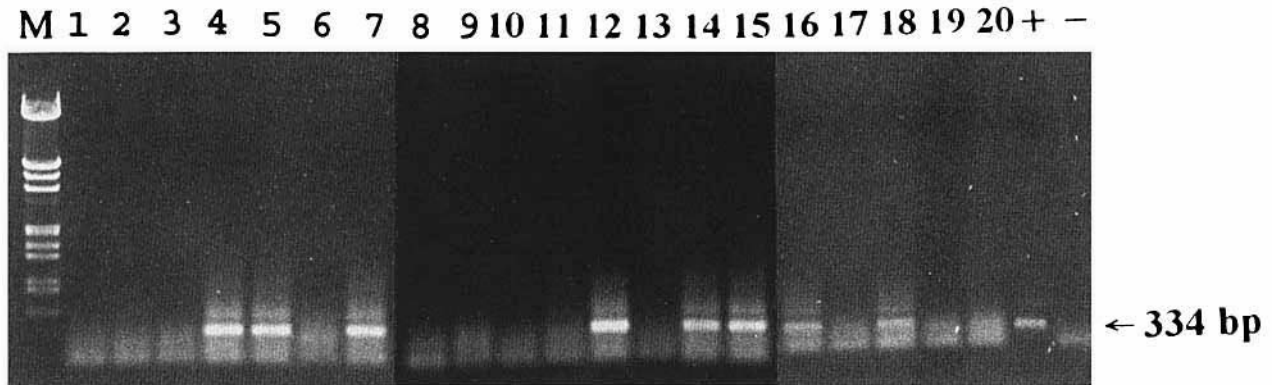


Fig. 1. Electrophoresis of PCR products from urine samples of autoimmune disease patients. The DNA fragments were amplified by using JB1 primer (5'-CCTCCACACCCTTACTTGAG-3') and JB2 primer (5'-GTGACAGCTGGCGAAGAACCATGGC-3'), flanking the promoter regions (334 bp) of JCV and BKV. After PCR, the products were separated

by 1.5% agarose electrophoresis and stained by ethidium bromide. Lane M, Lambda DNA digested with Eco RI and Hind III; lanes 1 through 20, patients' code numbers I₁ through I₂₀; lane +, 10 ng of JCV positive control DNA (pM1TCR1A) [Frisque et al., 1984]; lane -, negative control without adding any DNA.

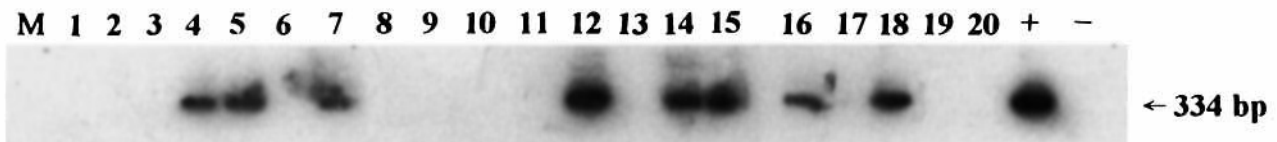


Fig. 2. Southern blot of JCV and BKV regulatory region. The DNA fragments of PCR products were electrotransferred onto nylon membrane after electrophoresis, probed with JCV and BKV specific ³²P-labeled oligonucleotide 5'-CCTAGGGAGCCAACCAGCTAACAGC-3',

and visualized by autoradiography. Lane M, Lambda DNA digested with Eco RI and Hind III; lanes 1 through 20, patients' code numbers I₁ through I₂₀; lane +, 10 ng of JCV positive control DNA (pM1TCR1A) [Frisque et al., 1984]; lane -, negative control without adding any DNA.

be 334 base pairs as shown in the control lane (Fig. 1, lane +). The possibility of false-negative was ruled out by the positive results of spiking 10 pg of the Mad-1 JCV genomic DNA in the negative samples for PCR (data not shown).

Southern Blotting

Southern blot analysis was employed to confirm that the 334 base-pair DNA fragments of PCR product from urine samples were the regulatory region of human polyomaviruses and to confirm that the negative samples did not contain small amount of DNA fragment which could not be detected by ethidium bromide staining after PCR. JBR-3 oligonucleotide probe which annealed at nucleotide 140 to 160 for JCV and 117 to 138 for BKV regulatory region was end-labeled with (³²P) ATP and hybridized with the nylon membrane containing the PCR products. The results of Southern blot showed that eight to 20 samples were JCV or/and BKV positive (Fig. 2, lanes 4, 5, 7, 12, 14, 15, 16, and 18). The results of Southern blot were the same as those of agarose electrophoresis with ethidium bromide staining shown in Figure 1. These results confirmed that the 334 base-pair DNA fragments generated by PCR were the regulatory region of human polyomaviruses and that the negative samples did not contain small amount of viral DNA fragments.

DNA Sequencing of the Regulatory Regions

The Southern blot positive samples were subsequently subjected to DNA sequencing to identify the viral strains present in the patients with autoimmune diseases in Taiwan. The DNA fragments of viral regulatory region of the PCR products were purified from low melting agarose gel and sequenced by *fmol* thermal cycling direct sequencing system.

DNA sequencing revealed that three different genotypes, Taiwan-1 (TW-1), Taiwan-2 (TW-2), and Taiwan-3 (TW-3), of JCV (Fig. 3) and two archetypal BKV, Taichung-1 (TC-1) and Taichung-2 (TC-2) (Fig. 4), were found in the human polyomavirus-positive urine samples. It was found that there were three copies of GGGAA located at nucleotide 30–34 (pentanucleotide A-1; PA-1), 198–202 (PA-2), and 218–222 (PA-3), and two copies of AAAGC located at nucleotide 188–192 (pentanucleotide B-1; PB-1) and 207–211 (PB-2) (Fig. 3) when examined for the CY archetype of JCV. When compared to the regulatory region of CY archetype, TW-1 JCV had PA-3 deletion. TW-2 had PA-2 and PA-3 deletions. TW-3 had PB-1 and PA-3 deletions. In addition, TC-1 BKV had two point alterations at nucleotide 52 (A→T) and 65 (T→C) and TC-2 BKV had five point alterations at nucleotide 52 (A→T), 65 (T→C), 199 (C→T), 205 (G→C), and 220 (C→G), and one base (G) insertion between

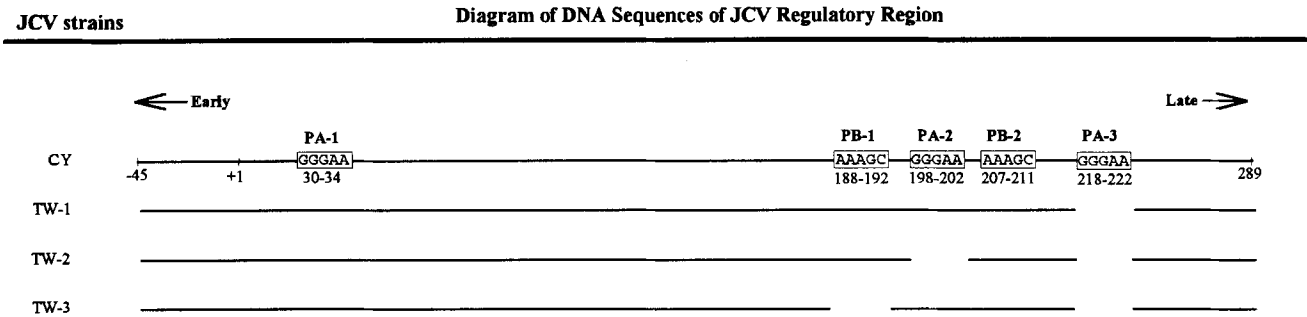


Fig. 3. Comparison of DNA sequences of CY, TW-1, TW-2, and TW-3 JCV regulatory regions. The DNA sequences of TW-1, TW-2, and TW-3 JCV regulatory region were sequenced from -45 to 289. CY archetype [Yogo et al., 1990] contains three copies of GGGAA pentanucleotide (PA-1, PA-2, and PA-3) and two copies of AAAGC pentanucleotide (PB-1 and PB-2). TW-1 has PA-3 deletion, TW-2 has PA-2 and PA-3 deletions, and TW-3 has PB-1 and PA-3 deletions at the regulatory region.

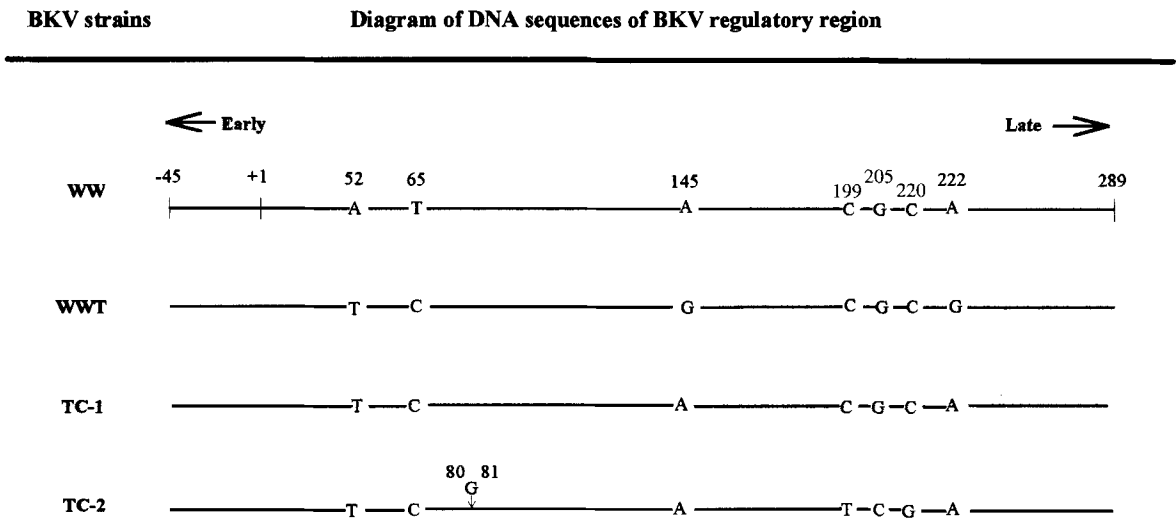


Fig. 4. Comparison of DNA sequences of WW, WWT, TC-1, and TC-2 BKV regulatory regions. The DNA sequences of TC-1 and TC-2 BKV regulatory region were sequenced from -45 to 289. Nucleotides 52, 65, 145, 199, 205, 220, and 222 are various among WW [Rubinstein et al., 1987], WWT [Sundsfjord et al., 1990], TC-1, and TC-2 BKV.

nucleotides 80 and 81 (Fig. 4) when compared to WW archetype of BKV [Rubinstein et al., 1987]. It is interesting that the altered nucleotides 52, 145, and 179 of TC-1 and TC-2 BKV (Fig. 4) were the hot spots for nucleotide variation in the regulatory regions of different BKV strains [Negrini et al., 1991]. Patient I₄ was infected by TW-2 JCV and TC-1 BKV. I₅ was infected by TW-2 JCV and TC-2 BKV. I₁₂ was infected by TW-3 JCV. I₁₄, I₁₅, I₁₆, and I₁₈ were infected by TW-1 JCV (Table I). There were no patients infected by BKV alone. Furthermore, based on densitometry of the DNA sequencing film of samples I₄, I₅, and I₇, the ratio of JCV and BKV DNA was approximately 1 to 1. It was about 40% of the examined urine samples JCV positive and 15% both JCV and BKV positive (Table II).

DISCUSSION

In our previous study, we have found that the JCV present in pregnant individuals in Taiwan were CY and

Taiwan-1 strains and BKV were Taichung-1 strain [Chang et al., 1995]. The JCV archetypal strain CY has been isolated from urine samples of nonimmunosuppressed individuals [Yogo et al., 1990]. The BKV and JCV shed in urine of pregnant women are predominately archetypal strain [Markowitz et al., 1991]. More recently, White et al. [1992] showed that the JCV present in kidney tissue of non-PML patients were archetypal but the JCV present in the brain tissue for both PML and non-PML patients were "PML-type" variant strains. These investigations indicate that the JCV may be present in kidney tissue of most healthy individuals with archetypal strain and that long-term immunosuppression may cause the genetic rearrangement of viral DNA and the infection of brain cells resulting in PML.

Three copies of GGGAA pentanucleotide-A (PA) are present within the regulatory region and located at nucleotide 30 to 34 (PA-1), 198 to 202 (PA-2), and 218 to 222 (PA-3) respectively and two copies of AAAGC

TABLE I. Infection of Human Polyomaviruses in Autoimmune Disease Patients

Patients codes	Immune diseases ^a	Medication drugs ^b	Periods of medication (month)	Strains of human polyomaviruses
I ₄	RA	OG, MTX, P	28	TW-2 JCV; TC-1 BKV
I ₅	SLE	HQ, MTX, P	27	TW-2 JCV; TC-2 BKV
I ₇	PM	Cys, P	5	TW-1 JCV; TC-2 BKV
I ₁₂	RA	HQ, MTX, P	4	TW-3 JCV
I ₁₄	RA	HQ, P	8	TW-1 JCV
I ₁₅	SS	None	—	TW-1 JCV
I ₁₆	RA	HQ, P	3	TW-1 JCV
I ₁₈	RA	HQ, D-P, P	15	TW-1 JCV

^aRA, rheumatoid arthritis; SLE, systemic lupus erythematosus; PM, polymyositis; SS, Sjogren's syndrome.

^bOG, oral gold; MTX, methotrexate; P, prednisolone; HQ, hydroxychloroquine; Cys, cyclosporine-A; D-P, D-penicillamine.

TABLE II. Summary of Human Polyomaviruses Genotypes in Autoimmune Diseases Patients in Taiwan

Virus	Positive (n = 20)	Genotypes
JCV	8 (40%)	TW-1, 62.5% TW-2, 25.0% TW-3, 12.5%
JCV + BKV	3 (15%)	TW-1 JCV + TC-2 BKV, 33.3% TW-2 JCV + TC-2 BKV, 33.3% TW-2 JCV + TC-1 BKV, 33.3%

pentanucleotide-B (PB) are located at nucleotide 188–192 (PB-1) and 207–211 (PB-2) of CY archetype (Fig. 3). In this study, we found three JCV strains, TW-1 with PA-3 deletion, TW-2 with PA-2 and PA-3 deletions, and TW-3 with PB-1 and PA-3 deletions, in the autoimmune disease patients. Interestingly, in our previous study [Chang et al., 1995], we found only CY and TW-1 JCV strains in pregnant individuals in Taiwan. Whether these JCV variants found in different patient groups are significant in the pathogenesis needs to be further investigated.

Tada and Khalili [1992] have found a brain-derived DNA binding protein LCP-1 (lytic control element-binding protein 1) which was able to bind the lytic control element (LCE) (AGGGAAGGGA) and to regulate the early gene expression of JCV lytic cycle in glial cells. Furthermore, Kumar et al. [1993] have shown that TGGAAAGCAGCCA was one of the nuclear factor 1 (NF-1) motifs important for glial cell-specific expression of JCV in differentiated embryonal carcinoma cells in vivo. More recently Kumar et al. [1994] have also shown that GGG regions in the LCE are required for glial cell-specific transcription. Amemiya et al. [1992] have found that nuclear factor 1 of glial cells was able to bind a specific site, site C (nucleotides 207–231), in the regulatory region of JCV, which contained AAAGC (PB-2) and GGGAA (PA-3), and it may involve gene regulation of JCV in glial cells. Based on this information, TW-1 TW-2, and TW-3 might be more inactive in glial cells since TW-1 had a copy of GGGAA deletion, TW-2 had two

copies of GGGAA deletion, and TW-3 had a copy of GGGAA and a copy of AAAGC deletion. However, the tissue tropism of these new strains needs to be further analyzed.

In this study, 5 of 11 RA patients, 1 of 3 SLE patients, 1 of 3 PM patients, and 1 of 2 SS patients were infected by JCV and BKV (Table I). These findings indicate that there is no correlation between the virus and the diseases. In addition, it also appears that there is no correlation between immunosuppressive drugs and the viruses, since those patients taking drugs were not infected by the same viruses (Table I). The most interesting factor is the duration of immunosuppression to the virus strains. I₄ and I₅ patients had the longest immunosuppression period among the human polyomavirus-positive patients in this study and both of them carried TW-2 JCV. The rest of the virus-positive patients had relatively shorter immunosuppression periods and they were infected by TW-1 JCV except for I₁₂, who was infected by TW-3. At this point, we are not able to conclude the correlation between the virus strains and the duration of immunosuppression. However, we will follow up those patients infected by human polyomaviruses to investigate if the long term immunosuppression could cause the variation of the virus strains.

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